New serologic findings in a patient with ulcerative colitis and a warm autoantibody

T.G. Lightfoot and L. Delia VanThof

IgG-RBC sensitization associated with serine proteases is the current prevailing hypothesis used to explain an uncommon phenomenon in which a positive DAT is obtained using the RBCs from a patient's clotted blood sample but a negative DAT is obtained when testing RBCs from the patient's unclotted sample. Similarly, the patient's serum but not plasma will also be reactive by IAT against all RBCs tested. The majority of patients demonstrating this phenomenon have had a history of ulcerative colitis but no signs of hemolytic anemia. A case of IgG-RBC sensitization associated with serine proteases and a warm autoantibody in a 14-year-old Hispanic girl with ulcerative colitis is reported. The patient was admitted for severe anemia (Hb, 6.9 g/dL). On admission, pretransfusion testing of the patient's serum and RBCs showed an ABO/Rh discrepancy between the forward typing and reverse grouping. The phenomenon of IgG-RBC sensitization associated with serine proteases was considered in the differential evaluation of the serum versus plasma typing discrepancy. To confirm the presence of the phenomenon of IgG-RBC sensitization associated with serine proteases, the plasma was clotted and converted to serum by the addition of thrombin. The initially nonreactive plasma was 2+ reactive when converted to serum. A warm autoantibody was also detected during the course of serologic evaluation. The patient was transfused with 2 units of incompatible RBCs with no adverse reaction observed.
***Immunohematology* 2009;25:160–164.**

Key Words: serine proteases, serum, plasma, ulcerative colitis

Background

In 1980, Garratty and colleagues[1] described and investigated an unusual phenomenon in which a patient's blood had a positive DAT in clotted samples but a negative DAT in anticoagulated samples. Since it was first recognized, there have been several theories put forward to explain this phenomenon. The current prevailing hypothesis used to explain the phenomenon is one proposed by Garratty in 1993.[2] Garratty suggested that the observed phenomenon was attributable to IgG-RBC sensitization associated with serine proteases. In 1993, Garratty performed a review of case reports for patients with this phenomenon. He found that 71 percent of patients had a history of ulcerative colitis or other inflammatory bowel diseases.[2] The proposed mechanism for the phenomenon of IgG-RBC sensitization associated with serine proteases is that an antibody in the patient's serum is directed against an epitope present on most serine proteases.[3] Theoretically, the generated complexes would then bind to RBCs. The reactivity observed in clotted serum is attributed to the generation of thrombin (a serine protease in its activated form) in the coagulation process. The blood group specificity of the antibodies involved in this phenomenon is usually undetermined. There have been three instances in which antibodies reacting in serum but not in plasma have been determined to have blood group specificities, and they are autoanti-Sc1,[4] autoanti-Kp^b,[5] and autoanti-c.[6] Ulcerative colitis is also associated with a positive DAT as a result of warm autoantibodies. The association of a positive DAT with ulcerative colitis was first described by Lorber et al. in 1955.[7] Autoimmune hemolytic anemia (AIHA) is an infrequent but serious complication of ulcerative colitis with a reported frequency of 0.7 percent.[8] The prevalence of a positive DAT in ulcerative colitis patients without progression to AIHA is unknown. To our knowledge, we report the first case in which both the phenomenon of IgG-RBC sensitization associated with serine proteases and a warm autoantibody are present concurrently in a pediatric patient with ulcerative colitis.

Case Report

The patient is a 14-year-old Hispanic girl who initially presented in August 2006 at the age of 12 with an 8-month history of bloody stools, abdominal pain, and weight loss. She was hospitalized and a clinical workup was initiated. On physical examination she was pale and lethargic. Abnormal laboratory findings included a Hb of 4.0 g/dL (normal range, 11.5–16 g/dL); Hct, 13.4% (37–47%); MCV, 54.4 fL (80–96 fL); MCH, 16.4 pg (27–32 pg); MCHC, 30.2 g/dL (32–36 g/dL); erythrocyte sedimentation rate, 35 mm/h (0–20 mm/h); reticulocyte count, 3.2% (0.5–1.5%); LDH, 233 U/L (105–333 U/L); bilirubin, 0.7 mg/dL (0.3–1.9 mg/dL); C-reactive protein, 1.61 mg/dL (1.0–3.0 mg/dL); positive anti-cardiolipin antibodies (negative); ferritin < 2 ng/mL (7–140 ng/mL); iron, 9 µg/dL (50–170 µg/dL); alkaline phosphatase, 54 IU/L (20–140 IU/L); and positive atypical perinuclear antineutrophil cytoplasmic antibodies (P-ANCA) with a titer of 1:1280 (negative). Stool culture for *Clostridium difficile* toxin was positive. Other laboratory findings included a WBC of 6.9 × 10^3/µL (4.8–10.8 × 10^3/µL) with a differential of 25% segmented neutrophils, 28% bands, 30% lymphocytes, 13% monocytes, 2% eosinophils, and 2% atypical lymphocytes. Platelet count was 344 × 10^9/L (150–450 × 10^9/L); prothrombin time, 15.3 seconds (< 10–15 seconds); activated partial thromboplastin

time, 28.9 seconds (< 35 seconds); INR, 1.20 (0.8–1.2); and fibrinogen, 395 mg/dL (200–400 mg/dL). A CT scan of her abdomen showed inflammation of the entire colon. No abnormalities of the small bowel were seen on an upper gastrointestinal series. She underwent a colonoscopy, which showed inflammation and friability of the mucosa. Colonic biopsies showed cryptitis and crypt abscesses to the hepatic flexure. The colonoscopic findings in conjunction with her elevated atypical P-ANCA laboratory findings confirmed a diagnosis of ulcerative colitis. She was treated with intravenous methylprednisolone, oral mesalamine, metronidazole, and 6-mercaptopurine. The patient had no signs or symptoms of AIHA and this was not considered as a cause of her anemia. The patient did have significant GI bleeding, which her treating physician determined to be the cause of her anemia. During this hospitalization, she received 4 units of packed RBCs to correct her anemia. During the next year she required several courses of steroids and she was started on infliximab (Remicade) approximately 14 months after her initial diagnosis. She was observed to develop hives that worsened on subsequent dosing. The infliximab therapy was discontinued and adalimumab (Humira) was instituted.

In February 2008, when the patient was 14 years old, she was found to have a Hb of 6.9 g/dL on routine monthly blood work. She was subsequently admitted to the hospital for further workup and evaluation. Initial serologic evaluation showed a positive DAT. Serum reactivity also was detected with all RBCs tested at antiglobulin phase using standard tube technique. The serologic evaluation was unable to be completed by the hospital, and the samples were sent to the immunohematology reference laboratory (American Red Cross, West Henrietta, NY) for evaluation of a positive DAT and antibody identification.

Immunohematologic Evaluation and Results

The results of initial pretransfusion testing showed an ABO discrepancy between the forward typing and the reverse grouping when testing the patient's RBCs and serum using standard tube technique according to manufacturer's instructions. The patient's RBCs forward typed as group A and the Rh type was D+. Test results obtained with the reverse-grouping cells were inconclusive owing to 4+ reactivity obtained with all reverse-grouping cells (A_1, A_2, B, O cells; Referencells, Immucor, Inc., Norcross, GA). Pretransfusion testing using RBCs from an EDTA sample showed a positive DAT, reactive with both anti-IgG and anti-C_3 (Immucor, Inc.). DAT testing was not performed on RBCs from a clotted sample as our reference laboratory does not routinely perform DAT testing on such samples. A cold autoagglutinin was initially thought to be present on the patient's RBCs because of the additional reactivity detected with all reverse-grouping cells tested. The positive DAT result obtained with anti-C_3 could also be attributable to the presence of a cold autoagglutinin. To determine whether a cold autoagglutinin was indeed present, the patient's serum was treated with 0.01 M DTT (Sigma-Aldrich, St. Louis, MO) to abolish IgM activity. No change in reactivity was noted when the patient's DTT-treated serum was tested against the reverse-grouping cells. The patient's neat serum and reverse-grouping cells were then warmed to 37°C before being added to the appropriately labeled test tubes. The test tubes were incubated at 37°C for 1 hour before being examined for agglutination. Using the prewarmed patient serum and reverse-grouping cells, there was no change noted in reactivity when retested; as such the presence of a cold autoagglutinin was excluded. The results obtained using these testing methods are shown in Table 1.

Table 1. ABO/Rh discrepancy detected using patient's serum

	Anti-A	Anti-B	Anti-A,B	Anti-D	Rh control	A_1 cells	A_2 cells	B cells	O cells
Patient's RBC/serum	4+	0	4+	4+	0	4+	4+	4+	4+
DTT-treated serum	NT	NT	NT	NT	NT	4+	4+	4+	4+
Prewarmed serum	NT	NT	NT	NT	NT	4+	4+	4+	4+

NT= not tested.

An additional patient sample was requested to confirm that the discrepant reactivity was in fact sample related and not attributable to a sample tube mix-up. The reactivity detected with the additional sample was identical to that of the original sample submitted, excluding the possibility of a sample tube mix-up.

Owing to the initial reactivity detected with the reverse-grouping cells, a selected cell panel was tested in a saline tube at 22°C to determine whether the reactivity was attributable to an IgM antibody. No antibody reactivity, however, was detected when this testing was performed using the patient's plasma. The same selected cell panel was then retested using the patient's serum, and all cells were noted to be 4+ reactive. Because discrepant reactivity was detected between the patient's serum and plasma, the patient's plasma was tested against the reverse-grouping cells, and no ABO discrepancy was detected. Using this method, the patient's blood type was determined to be A positive, which was consistent with the submitting hospital's historical records.

During the process of evaluating the serum versus plasma discrepancy, the possibility of IgG-RBC sensitization associated with serine proteases was considered. To further evaluate this hypothesis, the patient's plasma was clotted using thrombin (Sigma-Aldrich) to convert the plasma to serum. The plasma was initially nonreactive when tested against the group O reverse-grouping cells. Using the same

group O reverse-grouping cells, the converted serum was found to be 2+ reactive (see Table 2 for the test results obtained using the patient's plasma).

As stated earlier, there have been three instances in which antibodies reacting in serum but not in plasma have been determined to have blood group specificities.[4–6] The patient's RBCs tested positive for the Sc1 antigen; however, Sc1-negative RBCs were 4+ reactive when tested against the patient's serum in Micro Typing System (MTS) IgG gel (Ortho-Clinical Diagnostics, Raritan, NJ), thus excluding a possible IgG autoantibody with anti-Sc1 specificity. Autoanti-c was excluded based on the patient's phenotype; the patient's RBCs tested negative for the c antigen. Autoanti-Kp^b was not evaluated as this possibility was not known at the time of testing and was later identified during a further literature search after the completion of this workup.[5]

Table 2. ABO/Rh testing using patient's plasma

	Anti-A	Anti-B	Anti-A,B	Anti-D	Rh control	A1 cells	A2 cells	B cells	O cells
Patient's RBC/ plasma	4+	0	4+	4+	0	0	0	4+	0
Thrombin-treated plasma	NT	NT	NT	NT	NT	NT	NT	NT	2+

NT= not tested.

Because the patient's plasma was nonreactive when tested in a saline tube at 22°C, antiglobulin testing was performed using the patient's plasma. Initial testing was performed in both MTS IgG gel and LISS-AHG (Immucor, Inc.). The patient's plasma was 3+s reactive in both MTS IgG gel and LISS-AHG with three of three screening cells (Surgiscreen; Ortho-Clinical Diagnostics, Inc.) and two phenotypically similar cells. Autologous cells treated with EDTA-glycine acid (EGA) (Immucor, Inc.) to remove the IgG coating were also 3+ reactive. The patient's serum was also tested and found to be 4+ reactive with the same two phenotypically similar cells when tested in MTS IgG gel.

The pattern of reactivity detected when testing the patient's plasma suggested the presence of an IgG autoantibody. We were unable to determine whether the reactivity detected when testing the serum sample at antiglobulin phase was also attributable to a possible IgG autoantibody or interference of the serine protease antibody. A 3× alloadsorption was performed onto a phenotypically similar, R_1R_1 K– Jk(b–), glutaraldehyde-treated RBC to remove the IgG autoantibody reactivity and assess for the presence of underlying alloantibodies. No underlying alloantibodies were detected when testing the 3× alloadsorbed plasma in LISS-AHG. Alloantibodies to the major blood group antigens were ruled out by this testing. An IgG autoantibody was concluded to be in the patient's plasma because of:

1. Patient's positive DAT, with anti-IgG, detected using tube technique
2. Positive autocontrol, which consisted of the patient's RBCs treated to DAT negative tested against the patient's plasma in LISS-AHG

On February 14, 2008, the patient had a pretransfusion Hb of 6.9 g/dL and was transfused with 2 units of least-incompatible packed RBCs, attaining a posttransfusion Hb of 10.2 g/dL. There was no clinical or serologic evidence of an adverse reaction after transfusion.

Discussion

This report describes the presence of the phenomenon of IgG-RBC sensitization associated with serine proteases in a patient with ulcerative colitis. In this case, a positive DAT owing to the presence of a warm autoantibody was also present. To our knowledge this is the first published case in which both entities are concurrently present in a patient. Both of these entities may have a respective association with ulcerative colitis. In this case, the antibodies involved in both the phenomenon of IgG-RBC sensitization associated with serine proteases and the presence of a warm autoantibody were of the IgG class. The mechanism of the phenomenon of IgG-RBC sensitization associated with serine proteases is not completely understood. The initial hypothesis proposed for the phenomenon was that the patient had an IgG antibody to an activated coagulation factor that would not normally be present in plasma and that IgG immune complexes formed and attached to RBCs in vitro.[1] In 1993, Garratty,[2] performed a review of 28 patients with ulcerative colitis who manifested the IgG-RBC sensitization phenomenon associated with in vitro clotting as initially reported in 1980. In this review, the author observed that 10 of 11 IgG antibodies tested were of the IgG3 subclass. Further, the author found that the IgG-RBC sensitization phenomenon was not directly related to clotting. Garratty observed that a nonreactive plasma could be made reactive without clotting occurring by adding a variety of serine proteases (e.g., trypsin) to the plasma. Therefore the hypothesis was subsequently modified to suggest that the antibody in the patient's serum was directed against an epitope present on most serine proteases. This would include but would not be restricted to activated coagulation factors.[3]

In humans there are four broad classes of proteases, based on the mechanism they use to hydrolyze peptide bonds. Respectively, they are serine, cysteine, aspartic, and metalloproteases.[9] Of these classes, the serine proteases are the largest group, and their functions range from those attributable to nonspecific digestive enzymes such as trypsin, to more highly regulated functions such as blood coagulation (thrombin) and immune response (complement system). Nearly all serine proteases have amino acid sequence homology with pancreatic serine proteases. As a result,

serine proteases have very similar tertiary structures.[10] The maintenance of structural homology within the serine protease class would be consistent with Garratty's hypothesis regarding an antibody to an epitope commonly shared by serine proteases and his observation that a variety of serine proteases and not only those generated during the coagulation process were capable of inducing the phenomenon of IgG-RBC sensitization associated with serine proteases.[3]

Ulcerative colitis is a chronic inflammatory bowel disease that may be associated with extraintestinal immune features. Approximately 23 to 42 percent of patients with inflammatory bowel disease exhibit extraintestinal immunologic manifestations.[11] Immune hematologic features such as idiopathic thrombocytopenic purpura and AIHA have been reported in patients with ulcerative colitis.[8,12] Recently, the acquired form of thrombotic thrombocytopenic purpura (TTP) has also been described in patients with ulcerative colitis.[13] The most favored hypothesis for the pathophysiology of acquired TTP is that of an autoimmune process caused by autoantibodies that inhibit the activity of the von Willebrand factor–cleaving metalloprotease ADAMTS13.[14]

As the majority of the patients in whom the phenomenon of IgG-RBC sensitization associated with serine proteases has been observed have a history of ulcerative colitis or other inflammatory bowel disease, the phenomenon of IgG-RBC associated with serine proteases may similarly reflect another autoimmune extraintestinal immunologic manifestation of inflammatory bowel disease. The autoimmune mechanism previously described in the pathogenesis of acquired TTP (autoantibody to a protease) would also be a plausible model for the phenomenon of IgG-RBC sensitization associated with serine proteases.

A positive DAT in association with ulcerative colitis may occur as a result of the phenomenon of IgG-RBC sensitization associated with serine proteases or may be attributable to a warm autoantibody with possible autoimmune hemolytic anemia. The presence of both of these distinct entities in the same patient is a rare occurrence. Serologic investigation of the phenomenon of IgG-RBC sensitization associated with serine proteases may be quite challenging, and information regarding the patient's history of ulcerative colitis is beneficial. The clinical significance of the phenomenon of IgG-RBC sensitization associated with serine proteases is unknown. However, as best we are able to determine, it is not associated with accelerated RBC destruction in vivo. For those patients who have a positive serum and the phenomenon of IgG-RBC sensitization associated with serine proteases is believed to be present, serologic testing may be repeated with plasma. If the plasma is subsequently negative then compatibility testing may be performed using the plasma as per the *Standards* of the American Association of Blood Banks, which allows plasma to be used for compatibility testing.[3,15] Ulcerative colitis with a positive DAT and warm autoantibody is on rare occasion associated with hemolytic anemia and will require the appropriate transfusion recommendations. In such cases, incompatible units screened negative for possible underlying alloantibodies will need to be selected, as crossmatch units will appear incompatible because of the presence of the IgG autoantibody. The patient will need to be monitored closely for signs and symptoms of hemolysis after transfusion. In summary, although the phenomenon of IgG-RBC sensitization associated with serine proteases does not appear to be associated with accelerated RBC destruction, it is important that this phenomenon be considered and recognized to avoid needless delay in serologic workup and the provision of blood for the patient.

References

1. Garratty G, Davis J, Myers M, Nelson A, Pierce A, eds. IgG red cell sensitization associated with in vitro clotting in patients with ulcerative colitis (abstract). Transfusion 1980:20:646.
2. Garratty G. In vitro IgG sensitization of red cells associated with ulcerative colitis and serine proteases (abstract). Transfusion 1993;33(Suppl):22S.
3. Garratty G. In vitro reactions with red blood cells that are not due to blood group antibodies: a review. Immunohematology 1998;14:1–15.
4. Tregellas WM, Holub MP, Moulds JJ, Lacey PA. An example of autoanti-Sc1 demonstrable in serum but not in plasma (abstract). Transfusion 1979;19:650.
5. Moses BH, South SF, Ubbelohde M. Serum reactive, plasma nonreactive autoanti-Kel4 (Kp^b) (abstract). Transfusion 1991;31(Suppl):26S.
6. South SF, Davis K. Serum-reactive, plasma-nonreactive autoanti-c (abstract). Transfusion 1993;33(Suppl):22S.
7. Lorber M, Schwartz LI, Wasserman LR. Association of antibody-coated red blood cells with ulcerative colitis. Am J Med 1955;19:887–94.
8. Gumaste V, Greenstein AJ. Meyers R, Sachar DB. Coombs-positive autoimmune hemolytic anemia in ulcerative colitis. Dig Dis Sci 1989;34:1457–61.
9. Hart JP, Pizzo SV. α-Macroglobulins and kunins. In: Colman RW, Clowes AW, Goldhaber SZ, Marder VJ, George JN, eds. Hemostasis and Thrombosis: Basic Principles and Clinical Practice. 5th ed. Philadelphia, Pa: Lippincott Williams & Wilkins; 2006:395-407.
10. Perona JJ, Craik CS. Structural basis of substrate specificity in the serine proteases: a review. Protein Sci 1995:337–60.
11. Greenstein AJ, Janowitz HD, Sachar DB. The extraintestinal complications of Crohn's disease and ulcerative colitis. A study of 700 patients. Medicine 1976;55:401–12.
12. Zlatanic J, Korelitz BI, Wisch N, et al. Inflammatory bowel disease and immune thrombocytopenic purpura: is there a correlation? Am J Gastroenterol 1997;92:2285–8.

13. Baron BW, Jeon HR, Glunz C, et al. First two patients with ulcerative colitis who developed classical thrombotic thrombocytopenic purpura successfully treated with medical therapy and plasma exchange. J Clin Apher 2002;17:204–6.
14. Klaus C, Plaimauer B, Studt JD, et al. Epitope mapping of ADAMTS13 autoantibodies in acquired thrombotic thrombocytopenic purpura. Blood 2004;103:4514–19.
15. Friday J, ed. Standards for blood banks and transfusion services. 25th ed. Bethesda, MD: American Association of Blood Banks, 2008.

Thomas G. Lightfoot, MD (corresponding author), Associate Medical Director, American Red Cross Blood Services, NY-Penn Region, and Laurie Delia VanThof, MT (ASCP) SBB, Manager, Immunohematology Reference Lab/Stem Cell Lab, NY-Penn and NEPA Regions, 825 John Street, West Henrietta, NY 14586.